









Extremely low oxygen partial pressures for the storage of 'Royal Gala' apple

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ABSTRACT: The aim of this study was to evaluate the effect of storing 'Royal Gala' apple under extremely low oxygen partial pressures on their anaerobic metabolism, ethylene production and fruit quality after 9 months of storage plus 7 days of shelf life at 20 °C. The storage conditions tested were: 1.2 kPa O₂ + 2.0 kPa CO₂; 0.7 kPa O₂ + 1.5 kPa CO₂; 0.5 kPa O₂ + 1.2 kPa CO₂; 0.4 kPa O₂ + 1.2 kPa CO₂ and 0.25 kPa O₂ + 0.0 kPa CO₂. The fruit storage temperature was 1.0 °C (± 0.1). Fruit stored at 0.4 kPa O₂ + 1.2 kPa CO₂ maintained higher flesh firmness and a higher percentage of healthy fruit. The fruit stored under the 0.25 kPa O₂ + 0.0 kPa CO₂ condition had lower flesh firmness as well as a reduced percentage of healthy fruit and a high production of anaerobic metabolism compounds, such as ethanol and ethyl acetate, which may have caused fruit quality loss. The storage of 'Royal Gala' apple under 0.4 kPa O₂ + 1.2 kPa CO₂ is a technique that can be commercially applied to keep a better fruit quality after a 9 months storage period.

Key words: anaerobic metabolism, ethyl acetate, ethylene, *Malus domestica*, physiological disorders.

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INTRODUCTION

Brazil has a production of about 1.1 million tons of apples, mainly concentrated in the states of Rio Grande do Sul and Santa Catarina, where Rio Grande do Sul produced 490,000 tons in the 2017 harvest (Agapomi 2018). Amongst the main cultivars is the 'Royal Gala', discovered in New Zealand in 1971, from a mutation of the cultivar Gala (Camilo and Denardi 2006). This cultivar has the potential to meet the requirements of Brazilian consumers because it presents a uniform red coloration of the epidermis and sweet taste, which gives it a good value and commercial acceptance (Brackmann et al. 2012).

Since the production of apples is concentrated in a short period of the year, the use of appropriate storage techniques is very important, so the fruit can be supplied throughout the entire year (Brackmann et al. 2008). A widely used storage technique is the controlled atmosphere (CA), a method that consists of reducing oxygen (O₂) partial pressure and raising carbon dioxide (CO₂) in the storage chamber, thereby reducing fruit metabolism and extending the shelf life of the apple (Brackmann et al. 2013; Ceretta et al. 2010). However, several losses due to decay incidence and flesh breakdown occur in CA storage (Watkins 2006; Watkins 2008). The partial pressure of O₂ used during the storage is one of the most important factors in the CA storage. The reduction of O₂ to partial pressure that is too low (below 0.3 kPa O₂), may increase the production of volatile compounds such as alcohol, acetaldehyde and ethyl acetate, which in high concentrations can be harmful to the fruit, reducing the flesh firmness and increasing the occurrence of physiological disorders (Both et al. 2017).

The metabolic pathways of the anaerobic metabolism begin with glycolysis, which converts glucose into pyruvate, which is further converted into acetaldehyde, ethanol and ethyl acetate releasing CO₂ in this process (Yang and

Hoffman 1984), which is activated when there is an insufficient O₂ supply, which is the final acceptor of the electron transport chain (Zabalza et al. 2009).

One of the techniques additional to CA, in order to maintain higher quality of the fruit, is the extremely low oxygen (ELO) storage. This technique consists of storing the fruit at oxygen partial pressures above the anaerobic compensation point but lower than in CA, which reduces the metabolism and consequently the consumption of sugars and acids by cell respiration (Weber et al. 2013). The storage of apple under extremely low oxygen (ELO) partial pressures, when carried dynamically out, for example in dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA-CF), maintains higher quality of fruit when they are stored in a 0.4 kPa oxygen partial pressure in comparison to the conventional CA (1.0 kPa O₂ + 1.2 kPa CO₂) (Zanella et al. 2008; Weber et al. 2016). However, it is likely that extremely low partial pressures of oxygen (below 0.5 kPa O₂) used in a static atmosphere may have a good effect on apple quality maintenance.

The aim of this study was to evaluate the effect of storing 'Royal Gala' apple at extremely low oxygen partial pressures on the anaerobic metabolism, ethylene production and fruit quality after 9 months of storage followed by 7 days of shelf life at 20 °C.

MATERIAL E METHODS

'Royal Gala' apples, harvested in a commercial orchard located in Vacaria (RS – Brazil), were used in this trial. The selection of fruits, such as discard of small fruits, with defects or with mechanical lesions, for the composition of the experimental samples was performed after the fruits were harvested and transported to the Postharvest Research Center of the Federal University of Santa Maria.

The samples were then homogenized by size and each composed of 30 fruits. The experiment was conducted in a completely randomized design, containing five treatments with four replicates each. Three fruit samples were submitted to the initial analysis, presenting the following values for each parameter evaluated: soluble solids (SS) of 11.6%; flesh firmness of 83.8 N; respiration rate of 15.1 mg CO₂ kg⁻¹·h⁻¹; 6.1 of iodine-starch index. Evaluated using the method proposed by Streif (1984), with an index of 1 (all fruit with starch) to 10 (without starch); activity of the ACC oxidase enzyme of 30.5 ng C₂H₄ kg⁻¹·h⁻¹; titratable acidity of 4.8 meq 100 mL⁻¹ and ethylene production of 43.7 µg·kg⁻¹·h⁻¹. The following storage conditions were evaluated: CA at 1.0 kPa O₂ + 2.0 kPa CO₂ (conventional CA), ULO at 0.7 kPa O₂ + 1.5 kPa CO₂ and ULO at 0.5 kPa O₂ + 1.2 kPa CO₂ and ELO at 0.4 kPa O₂ + 1.2 kPa CO₂ and ELO at 0.25 kPa O₂ + 0.0 kPa CO₂. The fruits were stored in 230L CA rooms that were hermetically sealed and the respective treatments were established.

The fruit storage temperature was set at 1.0 °C (± 0.1) and relative humidity at 94% (± 2.0). The maintenance of moisture during storage was obtained with the aid of containers with calcium chloride that were placed inside the chamber (10 g·kg⁻¹ fruit), which absorbed excess moisture in the room.

Electronic thermostats were used for the daily monitoring of the temperature, along with a mercury thermometer that was inserted in the fruits kept in the cold room. After placing the fruits in the experimental CA rooms, the temperature was reduced to 5.0 °C on the first day and then it was daily reduced in 1.0 °C until the final storage temperature.

The atmospheres were installed after the fruits reached 1.0 °C. The oxygen partial pressures were obtained by flushing the CA rooms with nitrogen. The oxygen was reduced by the N₂ flushing gradually on the first day to 5.0 kPa. Thereafter, during the first storage week, the oxygen was reduced down to 1.0 kPa in all the storage conditions. Subsequently, the pre-established partial pressure for each oxygen storage condition was reduced by the fruits' respiration. The CO₂ partial pressure was obtained by the fruit respiration. A gas analyzer (ULTRAMAT-23, Siemens) was used to measure the gas partial pressures during the storage period. A gas control device with a software brand Valis (Lajeado, Rio Grande do Sul) was automatically programmed to make four daily readings of O₂ and CO₂, comparing the reading to the preset partial pressure (set point) of each treatment. If the CO₂ partial pressures were above the established set point, the excess was absorbed by an absorber with Ca (OH)₂ and if the O₂ partial pressure was below the expected, cold air was injected into the chamber.

After 9 months of storage, the fruits were removed from storage and kept at 20 °C, for shelf life, for 7 days. After this period the following parameters were evaluated:

- a. flesh firmness: part of the epidermis was removed from the equatorial regions of the fruits, and with the aid of a manual penetrometer (Effegi) with a 11 mm tip, the force required to insert in the fruit flesh was measured. The results were expressed in Newton (N);
- b. titratable acidity: was evaluated by titration with NaOH 0.1 mol·L⁻¹, of 10 mL of diluted juice in 100 mL of distilled water until 8.1 pH. The results of the determination were expressed in meq 100 mg⁻¹;
- c. soluble solids (SS): determined with the aid of a refractometer. The juice was obtained with samples of 30 fruits in each replicate and the results were expressed in %;
- d. flesh breakdown: All the 30 fruits of each sample were cut in half and then the presence of browning in the flesh was visually evaluated. The results were expressed in percentage;
- e. ethylene production: determined using 1,500 g of fruit kept hermetically sealed in glass jars with a volume of 5 L. The jars with fruits remained closed for about 1 h, then 2 gas samples were removed from the headspace with 1.0 mL syringe, which were then injected into a gas chromatograph (Varian, Star 3400 CX, Palo Alto, USA) fitted with a flame ionization detector (FID) and a Porapak N80/100 column, with column, injector and detector temperatures of 90, 140 and 240 °C, respectively;
- f. ACC oxidase activity: determined according to Bufler (1986). The data were expressed in ng C₂H₄ kg⁻¹·h⁻¹;
- g. decay incidence and pulp cracking: evaluated by counting fruit that presented any symptoms (lesion higher than 5 mm). After opening the chambers and exposing the fruits at 20 °C, the decay incidence was evaluated. The results were expressed as a percentage;
- h. mealiness: was determined by quantification of fruits that presented mealy pulp. Data were expressed as a percentage;
- i. acetaldehyde, ethanol and ethyl acetate: were determined according to Saquet and Streif (2008); a seedless flesh sample was excised from the equatorial region of the fruit with the aid of a Philips Walita centrifuge. After that, 10 mL juice was placed in a 20 mL sealed vial. The juice samples were warmed in a water bath at 70 °C and kept during 30 min. Afterwards, a 100 µL sample of the headspace was injected into a Dani gas chromatograph fitted with a capillary DN-WAX column. The temperatures of the injector and detector were 60 and 250 °C, respectively. The results were expressed in µL·L⁻¹;
- j. using the methodology of Mannapperuma et al. (1991), the internal ethylene concentration was determined using the methodology of Mannapperuma et al. (1991), in which two 1.0 mL samples of this air were collected and injected into the same gas chromatograph used for the quantification of ethylene production. The results were expressed in µg·L⁻¹;
- k. respiration rate: the same glass jars used for the evaluation of ethylene production was used for this assessment. The CO₂ released by the fruits was measured using an Isolcell electronic gas analyzer. The results were expressed in mg CO₂·kg⁻¹·h⁻¹.

The data matrix was scaled for each variable to obtain the same weight for all variables (mean = 0 and variance = 1) using the software Unscrambler X (version 9.7, CAMO / S, Trondheim, Norway). After this, a principal component analysis (PCA) was performed to show an overview of the results. Data and means were subjected to analysis of variance (ANOVA) and the Tukey's test, with 5% probability of error ($p < 0.05$), respectively.

RESULTS AND DISCUSSION

The plant hormone ethylene present in the form of gas is responsible for triggering the ripening process of fruit (Saltveit 1999; Li et al. 2002; Taiz and Zieger 2009). ACC oxidase and ACC synthase are essential enzymes for the ethylene biosynthesis pathway (Yang and Hoffman 1984; Wang et al. 2002). A reduction in the activity of the ACC oxidase enzyme was observed with the decrease of the oxygen partial pressures; the treatments with 0.5, 0.4 and 0.25 kPa of O₂ caused the

lowest activity of the enzyme (Fig. 1c). This reduction can be explained as a consequence of extremely low partial pressures of oxygen, where molecular oxygen becomes limited to the ACC oxidase activity. Moreover, this enzyme is responsible for ethylene synthesis and is activated or inhibited depending on the concentration of the anaerobic metabolism products (Yang and Hoffman, 1984; Beaulieu and Saltveit 1997). A high concentration of acetaldehyde and ethanol can inhibit the activity of the ACC oxidase and reduce the ethylene production by fruit (Beaulieu and Saltveit 1997; Pesis 2005; Asoda et al. 2009; Thewes et al. 2015; Weber et al. 2016; Both et al. 2017), which helps to explain the low ACC oxidase activity in the fruits stored at 0.4 and 0.25 kPa_{O₂}. There was no significant difference for titratable acidity and soluble solids (Fig. 1a and b) for the tested conditions, and there were no differences with the decrease of partial pressures of O₂. These results are in agreement with the results found by Brackmann et al. (2015).

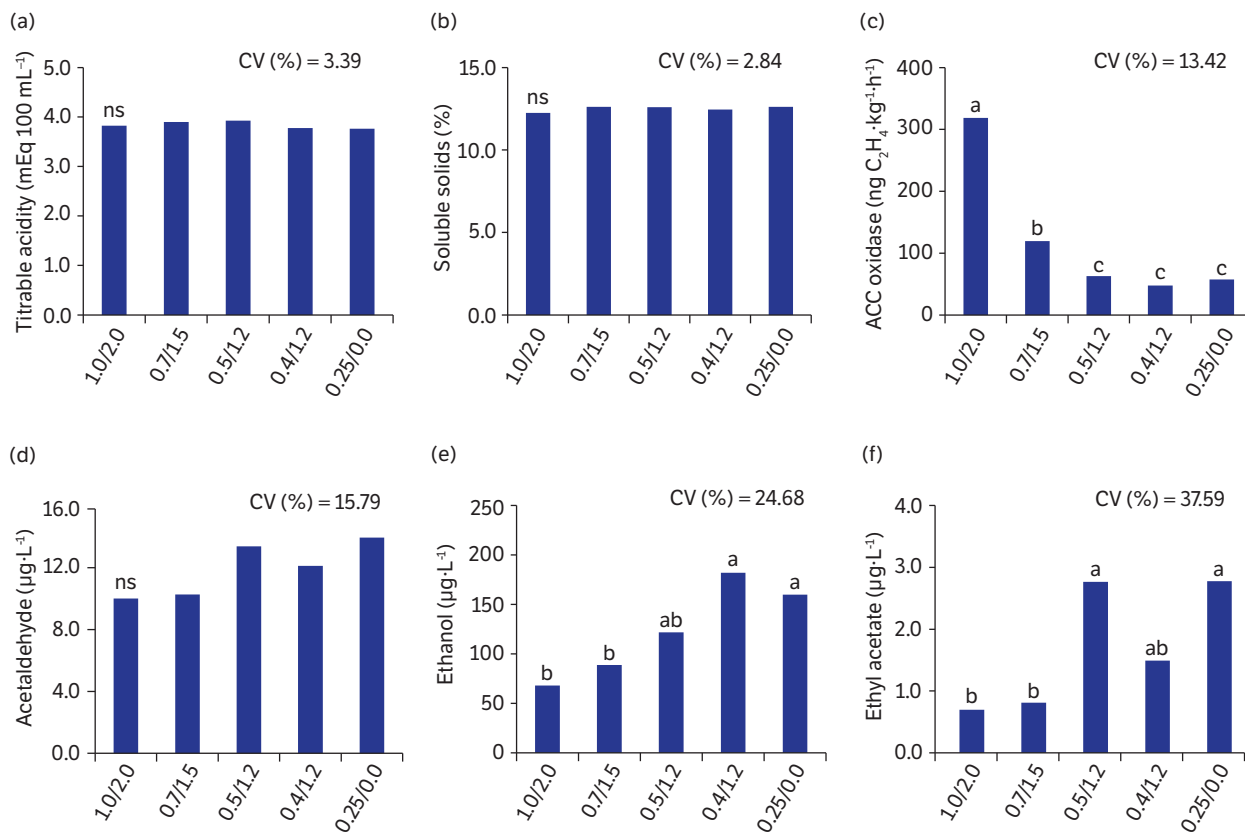


Figure 1. Titratable acidity (a), soluble solids (b), ACC oxidase enzyme activity (c), acetaldehyde (d), ethanol (e) and ethyl acetate (f) of 'Royal Gala' apples stored under ultra-low oxygen partial pressures. Averages followed by equal letter on the same parameter, do not differ by Tukey's test, at 5% of error probability.

The production and internal ethylene concentration increased significantly at the 7th day of shelf life in comparison at 0 days when apples were stored under CA with 1.0 kPa of O₂ + 2.0 kPa of CO₂ (Table 1). This finding is associated to a higher activity of the enzyme ACC oxidase measured in these fruits and conditions (Fig. 1c). However, fruits stored at O₂ partial pressures of 0.5, 0.4, and 0.25 kPa O₂ showed lower ethylene production and internal ethylene concentration (IEC) at chamber opening and after 7 days shelf life at 20 °C in comparison to the fruits stored under conditions of conventional CA with 1.2 kPa of O₂. This result is associated to the lower activity of the ACC oxidase measured under low oxygen (Fig. 1c).

The internal CO₂ concentration may be related to several factors, such as maturity stage, pulp structure, respiration rate and physiological disorders. According to Castro et al. (2008), high partial pressures of CO₂ inside the apple can cause oxidative stress and the production of free radicals leading to membrane change. The fruits stored at 0.7 kPa O₂ + 1.5 kPa CO₂

presented higher internal CO₂ concentration immediately after being removed from the rooms (Table 1). This result may have occurred due to the combined effect of the ULO condition, which induced the anaerobic metabolism pathway, and high CO₂ partial pressure, which differs from the other conditions with lower oxygen partial pressures. Furthermore, the fruits submitted to this treatment presented a higher respiration rate at chamber opening (Table 1), which may have favored the accumulation of CO₂ in the fruits. The lowest internal CO₂ concentration, in comparison to oxygen conditions lower than 1.0 kPa, was observed in the fruits stored with 0.25 kPa O₂ + 0.0 kPa CO₂. The lower accumulation of CO₂ in fruits stored under 0.0 kPa CO₂ may be related to the higher accumulation of fermentation products, which influenced the decrease in

Table 1. Internal ethylene concentration (IEC), ethylene production, internal carbon dioxide concentration (ICO₂) and respiration rate of 'Royal Gala' apples stored under extremely low oxygen partial pressures.

Conditions (O ₂ /CO ₂)	IEC (µg·L ⁻¹)		Mean	Ethylene production (µg C ₂ H ₄ ·kg ⁻¹ ·h ⁻¹)		Mean
	Shelf life at 20 °C			Shelf life at 20 °C		
	0 days	7 days		0 days	7 days	
1.0/2.0	6.32abB*	46.25aA	26.28	3.33aB	31.66aA	17.50
0.7/1.5	6.88aA	0.66bB	3.75	1.50aB	8.46bA	4.98
0.5/1.2	1.36bcA	0.20bA	0.78	0.57aA	1.22cA	0.90
0.4/1.2	1.27bcA	0.18bA	0.73	0.27aA	0.86cA	0.56
0.25/0.0	0.46cA	0.08bA	0.27	0.37aA	0.85cA	0.61
Mean	3.26	9.47	6.37	1.21	8.61	4.91
CV (%)	41.57			30.80		
	ICO ₂ (mg·100 mL ⁻¹)			Respiration rate (mg CO ₂ ·kg ⁻¹ ·h ⁻¹)		
1.0/2.0	4.97bcA	5.19aA	5.09	11.47bcA	12.11aA	8.82
0.7/1.5	8.01aA	4.62aB	6.32	14.94aA	9.66bB	9.93
0.5/1.2	5.72bA	4.95aA	5.34	13.27abA	8.00bB	8.67
0.4/1.2	6.03bA	4.60aB	5.32	12.15bcA	7.82bB	8.07
0.25/0.0	4.07cA	4.46aA	4.27	10.70cA	8.86bB	7.61
Mean	5.76	4.77	5.27	12.50	9.29	8.62
CV (%)	11.09			8.76		

*Averages followed by equal letter on the same parameter, lower case in the columns and uppercase in the lines, do not differ by Tukey's test, at 5%

metabolism, observed by the low respiration rate after being removed from the chamber. After 7 days at 20 °C, there was no difference among the assessed conditions (Table 1).

The 0.5 kPa O₂ + 1.2 kPa CO₂ and 0.7 kPa O₂ + 1.5 kPa CO₂ conditions presented higher respiration rates at chamber opening. However, at the 7th day there was a higher respiration rate in the fruits stored at 1.2 kPa O₂ + 2.0 kPa CO₂ partial pressure (Table 1). This may be associated to the ripening stage of fruit that were stored at higher oxygen partial pressures.

Regarding the acetaldehyde production, no difference was found among the evaluated treatments (Fig. 1d). However, there was a difference verified among the assessed conditions in regard to the ethanol production. The ethanol production increased as the O₂ decreased, wherein the fruit stored under the 0.4 and 0.25 kPa partial pressures of O₂ demonstrated a higher production of ethanol in comparison to the samples stored under 0.7 and 1.0 kPa of O₂ (Fig. 1e). This occurs due to the lack of molecular oxygen to serve as an electron acceptor in the respiratory chain (Lehninger et al. 2002). It may also be related to the increased activity of the alcohol dehydrogenase (ADH) under extremely low partial pressures of O₂ (0.4 and 0.25 kPa). The ADH enzyme acts on the conversion of acetaldehyde to ethanol using the NADH (nicotinamide adenine dinucleotide reduced) produced in glycolysis (Saquet and Streif 2008; Lee et al. 2012). Nanos et al. (1992) also observed similar results in pears stored under extremely low partial

pressure of oxygen (0.25 kPa), with high ethanol production and higher ADH activity, agreeing with the results obtained at the present work.

Fruit storage under partial pressures of 0.25 kPa of O_2 + 0.0 kPa of CO_2 maintained lower percentage of healthy fruits, flesh firmness, and flesh breakdown compared to the conditions of 0.4 kPa of O_2 + 1.2 kPa of CO_2 (Fig. 2c, e and f). Probably, the extremely low oxygen condition induced the anaerobic metabolism and formation of compounds such as acetaldehyde, ethanol and ethyl acetate, which are toxic substances, providing physiological disorders (Pedreschi et al. 2009). In agreement with Zanella (2003) and Pedreschi et al. (2009), the present work also observed higher anaerobic metabolism compounds like ethanol and ethyl acetate (Fig. 1e and f), which may directly influence the quality of 'Royal Gala' apples stored. Forney, Kalt and Jordan (2000) also observed higher ethyl acetate production of strawberry stored at CA, which was attributed to an *off flavor*. On the other hand, Both et al. (2014) did not find any difference between ultralow oxygen partial pressure

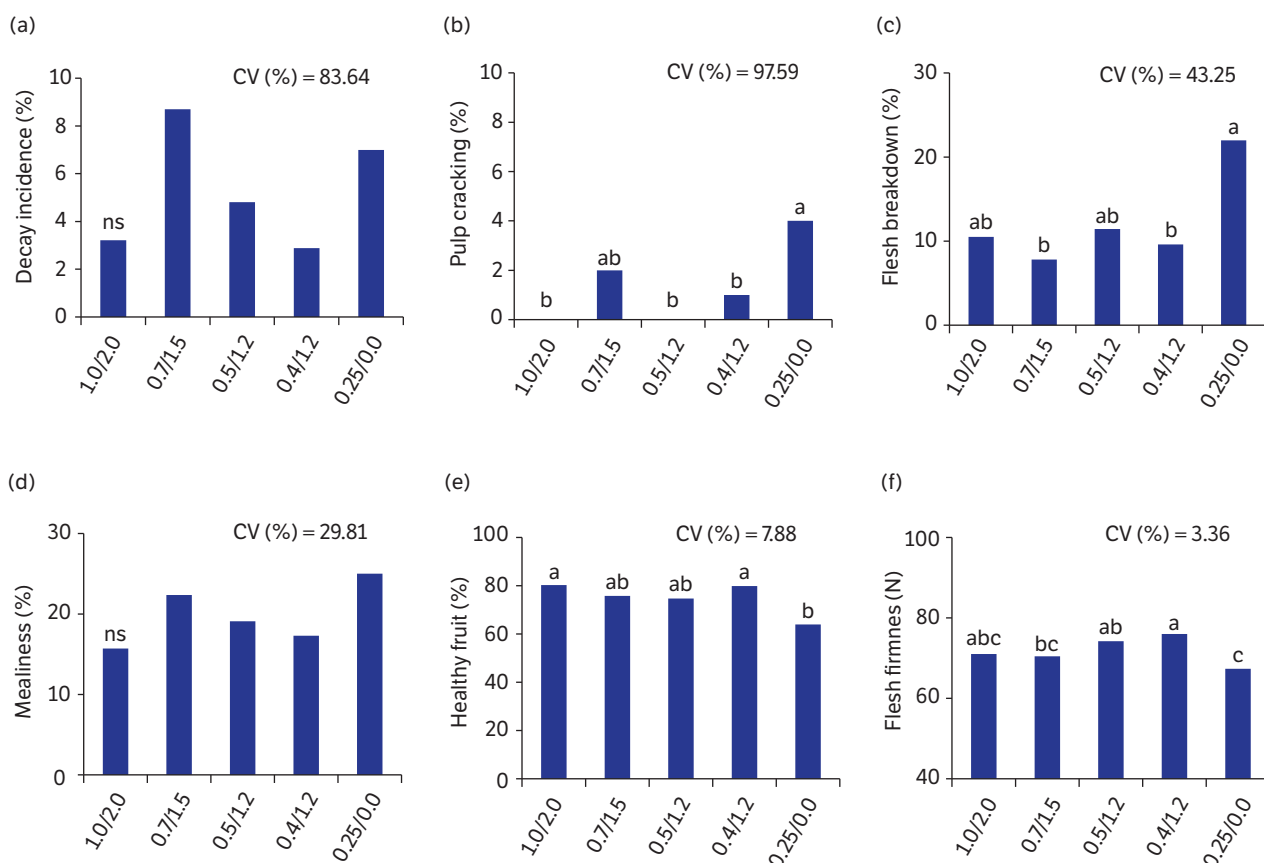


Figure 2. Decay incidence (a), pulp cracking (b), flesh breakdown (c), mealiness (d), healthy fruit (e) and flesh firmness (f) of 'Royal Gala' apples stored under extremely low oxygen partial pressures. Averages followed by equal letter on the same parameter do not differ by Tukey's test, at 5% of error probability.

(0.5, 0.7 and 0.8 kPa) and CA (1.0 kPa O_2) on the anaerobic metabolism compounds concentration after eight months of storage of 'Royal Gala' apples.

The principal component analysis (PCA) (Fig. 3a and b) shows the degree of correlation among the storage conditions and the analyzed parameters. The storage condition with 0.4 kPa O_2 + 1.2 kPa CO_2 presents a good correlation with the flesh firmness and healthy fruit. Flesh firmness was not associated with the ethylene production. Ethylene acts on the expression of enzymes involved with the cell wall degradation, such as polygalacturonase, pectin methylesterase, xyloglucan endotransglucosylase, cellulases, and others (Hiwasa et al. 2003; Prasanna et al. 2007). It was observed that as the oxygen partial pressures decreased (Table 1), ethylene production was reduced, which reflects in higher flesh firmness

(Fig. 2f). Thewes et al. (2015) have also verified in ‘Galaxy’ and ‘Royal Gala’ apples stored under ULO and DCA-CF a higher flesh firmness in comparison to conventional controlled atmosphere (CA). However, when the fruits were stored under 0.25 kPa O₂ + 0.0 kPa CO₂, in a static form, the flesh firmness was lower. This finding could be related to the damage due to accumulation of anaerobic metabolism compounds. However, when ‘Galaxy’ and ‘Royal Gala’ apples were in a DCA storage where the oxygen partial pressure was monitored by the respiratory quotient (DCA-RQ), and possessing an average O₂ near to 0.25 kPa, flesh firmness maintenance was observed (Thewes et al. 2017; Both et al. 2017).

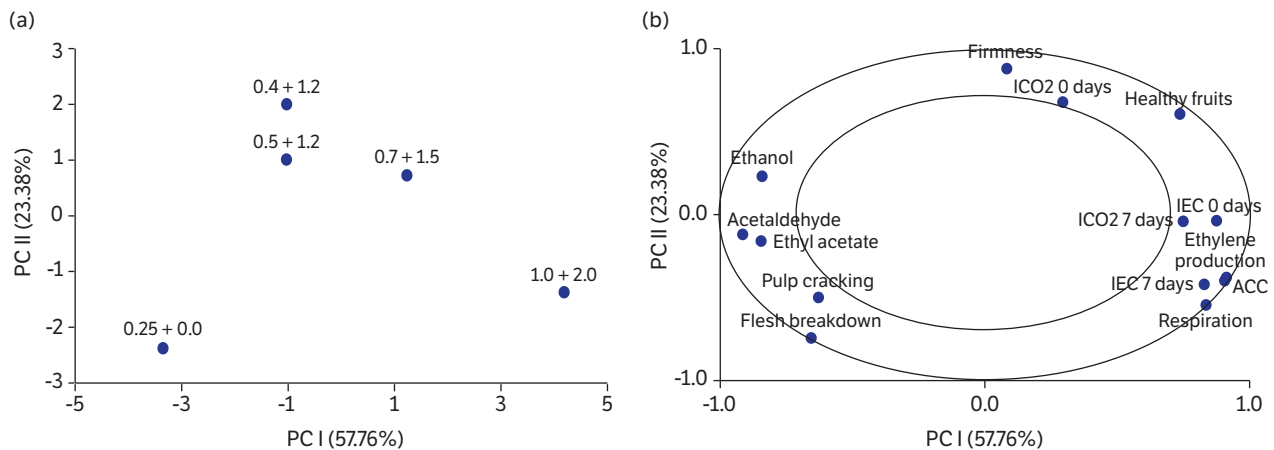


Figure 3. Scores (a) (treatments) and correlation loadings (b) (variables) of ‘Royal Gala’ apple stored under 1.0 kPa O₂ + 2.0 kPa CO₂, 0.7 kPa O₂ + 1.5 kPa CO₂, 0.5 kPa O₂ + 1.2 kPa CO₂, 0.4 kPa O₂ + 1.2 kPa CO₂ and 0.25 kPa O₂ + 0.0 kPa CO₂ during 9 months plus 7 days of shelf life at 20 °C.

In the PCA, a correlation between flesh breakdown and pulp cracking can also be noticed with the higher concentrations of acetaldehyde and ethyl acetate (anaerobic metabolism products accumulation) in the extremely low oxygen condition of 0.25 kPa O₂ + 0.0 kPa CO₂ (Fig. 3a and b). This can be explained by the rapid decrease of oxygen, from an aerobic to anaerobic level, which results in an accumulation of toxic volatile substances and, consequently flesh breakdown, characterized as physiological disorders (Pedreschi et al. 2009) and the pulp cracking. Ceretta et al. (2010) reported that the storage of ‘Gala’ apple at O₂ conditions under 0.8 kPa at 0 °C can increase the incidence of pulp cracking. In contrast, Weber et al. (2011) verified in ‘Royal Gala’ apples stored under ULO 0.6 kPa O₂ at a temperature of 1 °C lower occurrence of flesh breakdown. Therefore, it can be affirmed that there is a limit of reduction of the oxygen partial pressure under static CA, especially when the reduction of oxygen is fast at the beginning of the storage period (Fig. 2b and c), which below this limit, impair the quality of the fruits.

CONCLUSION

The 0.4 kPa O₂ + 1.2 kPa CO₂ storage condition was efficient in maintaining the quality of ‘Royal Gala’ apples stored for 9 months followed by 7 days at 20 °C allowing lower ethylene production, higher flesh firmness, lower pulp cracking and lower flesh breakdown occurrence.

The storage in the static conditions of 0.25 kPa O₂ + 0.0 kPa CO₂ did not maintain the quality of the fruits, presenting higher incidence of pulp cracking and flesh breakdown, thus these gas partial pressures are not recommended for storing ‘Royal Gala’ apple during 9 months in a static CA.

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